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Inhibition by guanosine cyclic monophosphate (cGMP) analogues of uptake of [^{3}H]3′,5′-cGMP without stimulation of ATPase activity in human erythrocyte inside-out vesicles

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Abstract

The cellular extrusion of guanosine 3′,5′-cyclic monophosphate (3′,5′-cGMP) is a unidirectional ATP-dependent process that is inhibited by probenecid, a non-selective transport inhibitor of organic anions. In the present study, various cGMP analogues were tested for their ability to inhibit 3′,5′-cGMP efflux and stimulate the cGMP-selective ATPase in human erythrocytes. The difference in uptake of 1 μM [^{3}H]3′,5′-cGMP to inside-out vesicles in the presence and absence of 1 mM ATP at 37° was defined as active transport. Two ATP-dependent components were detected for unlabelled 3′,5′-cGMP (0.01–100 μM) with respective K_i of 1.3 ± 0.2 and 280 ± 50 μM (mean ± SEM, N = 3). The high-affinity transport was inhibited by the analogues with a typical pattern: Rp-monophosphorothioate guanosine 3′,5′-cyclic monophosphate (Rp-cGMPS) > 3′,5′-cGMP > 2′-*O*-monobutyryl guanosine 3′,5′-cyclic monophosphate (*O*-mb-cGMP) ≈ N^2-monobutyryl guanosine 3′,5′-cyclic monophosphate (*N*-mb-cGMP) ≥ N^2,2′-*O*-dibutyryl guanosine 3′,5′-cyclic monophosphate (Db-cGMP) ≈ 8′-bromo guanosine 3′,5′-cyclic monophosphate (Br-cGMP) ≈ Guanosine 2′,3′-cyclic monophosphate (2′3′-cGMP) > Sp-monophosphorothioate guanosine 3′,5′-cyclic monophosphate (Sp-cGMPS). A concentration-dependent inhibition was found for the low-affinity transport, but no distinct order of potency was identified. Analysis according to Lineweaver–Burk of active [^{3}H]3′,5′-cGMP transport (0.2–2 μM) gave a K_m value of 1.5 ± 0.1 μM (mean ± SEM, N = 3). The presence of 10 μM cGMP analogues did not change the ordinate intercept, but made the slopes steeper with a typical order: Rp-cGMPS > 3′,5′-cGMP > *N*-mb-cGMP ≈ *O*-mb-cGMP ≈ db-cGMP ≈ 8-Br-cGMP > 2′,3′-cGMP > Sp-cGMPS. Only 3′,5′-cGMP and 2′,3′-cGMP were able to activate the cGMP-specific ATPase, 640 ± 200% and 430 ± 160% (mean ± SEM, N = 5) above basal levels, respectively. The present data show that the binding is less selective than ATPase activation of the cellular cGMP transport system.

Keywords: cGMP transport; ATP; ATPase specificity.

1. Introduction

Cyclic GMP is extruded from cells by an ATP-dependent transporter [1,2], recently identified as MRP5 [3]. The basic concept of such a transporter is that a sequence of functional steps is needed: (a) recognition of cGMP; (b) translocation of cGMP to outside of the cell; and (c) release of cGMP through a marked lowering of the binding affinity. The observations that high cAMP concentrations were needed to prevent cGMP extrusion [2,3] indicate that the transporter exhibits a considerable degree of selectivity. On the other hand, less structurally related compounds such as probenecid, verapamil, and forskolin inhibited the efflux in a competitive manner [2]. Specific inhibitors of cGMP phosphodiesterases such as zaprinast and sildenafil [3] inhibited the transport with high affinity, whereas high concentrations (>100 μM) of non-specific phosphodiesterase inhibitors (theophylline and isobutylmethyl xanthine) were needed to inhibit transport [2]. The question of selectivity is further complicated by the fact that glutathione conjugates interfere with the cGMP transport [4,5]. The purpose of the present

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Abbreviations: 3′,5′-cGMP, guanosine 3′,5′-cyclic monophosphate; 2′,3′-cGMP, guanosine 2′,3′-cyclic monophosphate; *N*-mb-cGMP, N^2-monobutyryl guanosine 3′,5′-cyclic monophosphate; *O*-mb-cGMP, 2′-*O*-monobutyryl guanosine 3′,5′-cyclic monophosphate; Db-cGMP, N^2,2′-*O*-dibutyryl guanosine 3′,5′-cyclic monophosphate; Br-cGMP, 8′-bromo guanosine 3′,5′-cyclic monophosphate; Rp-cGMPS, Rp-monophosphorothioate guanosine 3′,5′-cyclic monophosphate; Sp-cGMPS, Sp-monophosphorothioate guanosine 3′,5′-cyclic monophosphate; 3′,5′-cAMP, Adenosine 3′,5′-cyclic monophosphate; and MRP, multidrug resistance protein.

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study was to obtain more information about the selectivity of the cGMP pump. The ability to recognize various cGMP analogues and to stimulate ATPase activity was studied.

2. Materials and methods

2.1. Chemicals

[^{3}H]3′,5′-cGMP (sp. act. 16–18 Ci/mmol) was obtained from Amersham Pharmacia Biotech AB, and 3′,5′-cGMP, 2′,3′-cGMP, *N*-mb-cGMP, *O*-mb-cGMP, Db-cGMP, Br-cGMP, GMP, ammonium molybdate, disodium-ATP, SDS, and sodium metaarsenite were from Sigma Chemical Co. Rp-cGMPS and Sp-cGMPS were purchased from Biolog Life Science Institute. All other chemicals were of analytical grade.

2.2. Preparation of inside-out vesicles

Venous blood was sampled in 10-mL EDTA vacuum tubes (Vacutainer^R, Becton Dickinson), and the inside-out vesicles were prepared and isolated as described previously [4]. The vesicles were finally resuspended in 0.7–0.8 mL PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) for transport studies and in 10 mM Tris–HCl, 150 mM NaCl (pH 7.5) for the studies of ATPase activity and sidedness. The degree of sidedness of inside-out vesicles was virtually identical to that observed previously [2]. Due to a marked fall in transport activity within a few days, the experiments were performed with freshly prepared vesicles.

2.3. Transport studies

The analogues were co-incubated with [^{3}H]3′,5′-cGMP for 120 min at 37°. The radiochemical purity of [^{3}H]3′,5′-cGMP taken up by the vesicles was examined with thin-layer chromatography and with the same results as previously reported [1].

2.4. ATPase activity assay

The ATPase activity was determined by measuring the release of inorganic phosphate from ATP by a colorimetric method, basically as described by Chifflet [6]. The experiments were performed at 37° where the incubation mixture (200 μL) included 10 mM Tris–HCl, 150 mM NaCl (pH 7.5), inside-out vesicles (10–30 μg of protein), 2 mM $MgCl_2$, 1 mM ATP, 1 mM ouabain, and 2 mM EGTA with or without 2 μM 3′,5′-cGMP or its analogue. After 120 min. at 37°, the reaction was stopped by the addition of 200 μL of 12% (w/v) SDS. The color development was initiated by addition of 400 μL ascorbic acid (6% [w/v]) in HCl (1 M) and ammonium molybdate (1% [w/v]). The products were stabilised after 10 min by adding 600 μL sodium citrate (2% [w/v]), sodium metaarsenite (2% [w/v]) and acetic acid (2% [v/v]). The absorbance of each sample at 850 nm was measured in Pharmacia LKB-Ultrospec III. Inorganic phosphate released was measured in the absence and presence of 3′,5′-cGMP/cGMP analogues.

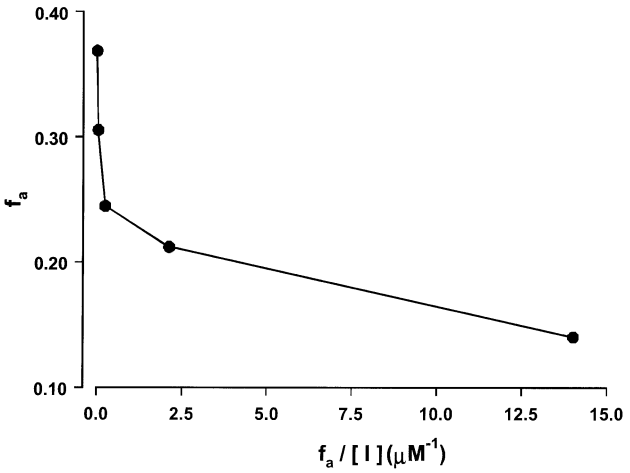

Fig. 1. Inhibition of active transport of [^{3}H]3′,5′-cGMP into inside-out vesicles by unlabeled 3′,5′-cGMP. Labeled (1 μM) and unlabeled (10 nM–100 μM) 3′,5′-cGMP were co-incubated for 120 min at 37°. The data were analysed in Hofstee inhibition plots [8]. In this transformation, the fraction of radioligand (fa) displaced is plotted against the fraction of radioligand displaced (fa) divided by the inhibitor concentration [I]. The results are presented as mean values of three separate experiments.

2.5. Protein concentrations

Protein concentrations were determined by the Coomassie brilliant blue method [7] with reagents from Bio-Rad Laboratories and BSA from Sigma Chemical Co. as standards.

3. Results

3.1. Inhibition of low and high K_m transport of [^{3}H]3′,5′-cGMP

Active transport into inside-out vesicles was defined as the difference in accumulation of [^{3}H]3′,5′-cGMP in the presence and absence of 1 mM ATP. Unlabeled 3′,5′-cGMP caused a concentration-dependent reduction in the active transport of [^{3}H]3′,5′-cGMP into inside-out vesicles from human erythrocytes. When data were plotted according to Hofstee [8], two components were detected (Fig. 1). Analysis according to Chou [9] gave apparent K_is of 1.3 and 280 μM. The non-cyclic nucleotide GMP did not influence the transport of [^{3}H]3′,5′-cGMP (data not shown). Hofstee analysis [8] showed that cGMP analogues inhibited both high- and low-affinity transport in a concentration-dependent manner. The transport with low K_m value repeatedly showed an identical pattern in all experiments: Rp-

Table 1
Inside-out vesicles exposed to 1 μM [^{3}H]-cGMP and cGMP analogues with concentrations from 10 nM to 100 μM for 120 min at 37°

Inhibitor	High affinity K_i (μM)	Low affinity K_i (μM)
Rp-cGMPS	0.6 ± 0.05	250 ± 170
3′,5′-cGMP	1.3 ± 0.2	280 ± 50
O-mb-cGMP	2.4 ± 0.1	310 ± 70
N-mb-cGMP	2.5 ± 0.2	290 ± 70
Db-cGMP	3.4 ± 0.4	310 ± 30
8′-br-cGMP	3.9 ± 0.6	320 ± 90
2′,3′-cGMP	4.1 ± 0.3	300 ± 40
Sp-cGMPS	13.5 ± 2.3	270 ± 60
GMP	No inhibition	No inhibition

The data from three separate experiments were analysed in Hofstee inhibition plots [8]. The results are presented as means ± SEM.

cGMPS > 3′,5′-cGMP > *O*-mb-cGMP ≈ *N*-mb-cGMP ≥ Db-cGMP ≈ Br-cGMP ≈ 2′3′-cGMP > Sp-cGMPS. Table 1 shows the stereoselective inhibition of high-affinity transport with Rp-cGMPS to be approximately 20-fold more potent than Sp-cGMPS. The inhibition of low-affinity transport of [^{3}H]3′,5′-cGMP by analogues showed no distinct pattern (Table 1).

3.2. Type of inhibition

Fig. 2 shows a Lineweaver–Burk plot with concentrations from 0.5 to 5 μM of [^{3}H]3′,5′-cGMP. Analysis of the plot resulted in a K_m of 1.5 μM and a V_{max} of 180 fmol/min/mg protein (mean ± SEM, N = 3). In the presence of cGMP analogues, virtually identical ordinate intercepts were found with the same order of apparent K_m values in each of three separate experiments: Rp-cGMPS > 3′,5′-cGMP > *N*-mb-cGMP ≈ *O*-mb-cGMP ≈ Db-cGMP ≈ Br-cGMP > 2′,3′-cGMP > Sp-cGMPS (Table 2).

3.3. ATPase activity

The transport of 3′,5′-cGMP is dependent on ATP hydrolysis [2]. The present experiments showed that 2 μM 3′,5′-cGMP caused a time-dependent linear accumulation of inorganic phosphate (linear regression: y = 0.064 nmol/mg protein/min * x − 0.85 nmol/mg protein, r^2 = 0.99). The total ATPase (basal plus cGMP-stimulated) activity was distinctly different from basal activity with respective values of 7.5 ± 2.1 and 0.8 ± 0.7 nmol/mg protein (mean ± SEM, N = 4) after 120 min. Cyclic 3′,5′-GMP and, to a lesser extent, 2′,3′-cGMP stimulated ATPase activity. A concentration of 2 μM of the cyclic nucleotides increased the ATPase activity by 640 ± 200 and 430 ± 160% (mean ± SEM, N = 5) above the basal level, respectively. The other analogues were unable to stimulate the ATPase activity (data not shown).

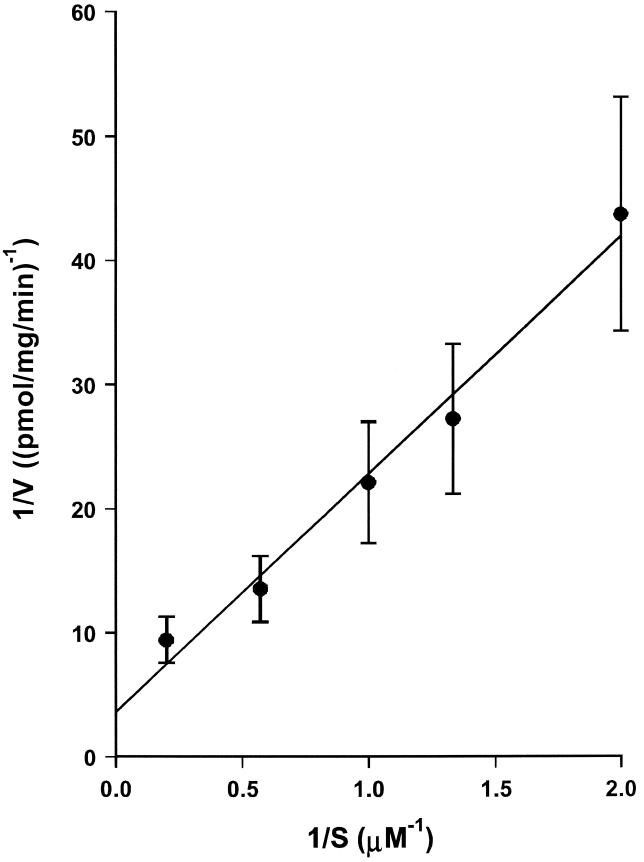

Fig. 2. Lineweaver–Burk plot of active transport of [^{3}H]3′,5′-cGMP into inside-out vesicles with concentrations from 0.5–5 μM. The vesicles were incubated for 120 min at 37°. The results are presented as mean values ± SEM of three separate experiments.

4. Discussion

In the last decade, an emerging number of ABC (ATP-Binding-Cassette) transporters has been characterized. Members of this transporter family have been identified in nearly all living cells. Within the sub-family of MRP, MRP5 was recently identified as the transporter of cGMP and other nucleotides [3,10]. Previous biochemical studies of the cGMP efflux pump showed that transport was dependent on ATP [1] and ATP hydrolysis [2] with magnesium as activator [4,5,11]. An ATPase inhibitor profile similar to that of the so-called M-type ATPases such as P-glycoprotein [12] was also shown [11]. The present study confirms the existence of a cGMP-specific ATPase that appeared to display considerable selectivity. Except for 3′,5′-cGMP and 2′,3′-cGMP, the tested cGMP analogues and GMP were unable to stimulate ATPase activity.

Biological specificity resides in the ability of macromolecules to discriminate between small molecules. The observation of a stereoselective inhibition (Rp-cGMPS vs Sp-cGMPS) suggests that spatial requirement is important. In this context, it is interesting to note that Rp-cGMPS is an inhibitor, whereas Sp-cGMPS acts as an activator of protein kinase G [13]. The ability to inhibit [^{3}H]3′,5′-cGMP trans-

Table 2
Inside-out vesicles incubated for 120 min at 37° with five concentrations (from 0.5 to 5 μM) of [^{3}H]cGMP in the absence or presence of cGMP analogues (10 μM)

Unlabelled substance	Apparent K_m (μM)	V_{max} (fmol/min/mg protein)
None	1.5 ± 0.10	180 ± 56
Sp-cGMPS	1.8 ± 0.15	205 ± 38
2′,3′-cGMP	2.0 ± 0.13	176 ± 50
8′-br-cGMP	2.3 ± 0.14	176 ± 57
Db-cGMP	2.4 ± 0.08	165 ± 46
O-mb-cGMP	2.5 ± 0.17	187 ± 55
N-mb-cGMP	2.6 ± 0.21	165 ± 47
3′,5′-cGMP	2.7 ± 0.34	165 ± 19
Rp-cGMPS	3.7 ± 1.3	181 ± 49

The data from three separate experiments were analysed in Lineweaver–Burk plots. The results are presented as means ± SEM.

port in a competitive manner made the cGMP analogues potential substrates for the cGMP pump. However, the absence of ATPase stimulation, with the exception of 3′,5′-cGMP and 2′,3′-cGMP, shows that these compounds have affinity for a common recognition binding site, but lack the spatial characteristics needed to activate the ATPase. The non-cyclic analogue, GMP, showed neither inhibition of transport nor ATPase stimulation. Only certain molecular volumes and tertiary structures make it possible for the substrate to fit into the substrate-binding site(s) of P-glycoprotein [14], and it has been suggested that overlapping stimulatory and inhibitory drug-binding sites exist within P-glycoprotein [15]. The present observation is in agreement with the recent report that 10 μM Db-cGMP and Br-cGMP inhibited [^{3}H]3′,5′-cGMP transport by about 30–40% [3]. Marked selectivity has also been observed towards cAMP. In a previous study, 100 μM cAMP reduced the cGMP transport by about 15% [2]. A recent study reported cAMP to be somewhat more potent, with 500 μM inhibiting about 65% of cGMP transport by MRP5 [3]. Leukotriene C_4 is a substrate for members of MRP family [16], but does not inhibit cGMP transport [3–5].

We have previously shown that low and high K_m ATP-dependent saturable transport exists for 3′,5′-cGMP in human erythrocytes [1]. The present study confirmed this observation with similar K_m and V_{max} values for 3′,5′-cGMP transport. However, a marked difference was observed in the selectivity. The cGMP analogues inhibited the low K_m transport with a typical order of potency, whereas such an order of potency was absent for the high K_m process. Matching K_m values suggest that the cGMP high-affinity pump is identical to MRP5 [3]. Immunoblot analyses performed on human erythrocyte membranes showed the presence of MRP1 [17] and MRP5 [3]. However, MRP1 is not a cGMP transporter since cGMP did not inhibit transport of LTC_4 [4,5]. The non-specific high K_m transport may reflect activity of a different low-affinity and high-capacity transport system. In agreement with this, overlapping substrate specificities were observed for the low K_m and high K_m components of *S*-dinitrophenyl-glutathione uptake by inside-out vesicles of human erythrocytes [18].

The present study shows the existence of a selective transport route for 3′,5′-cGMP across the red cell membrane. The selectivity appears to reside in two functional steps of the transporter: (a) competition for a recognition binding site; and (b) the ability to stimulate ATPase activity and thereby initiate transport.

Acknowledgments

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